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Organotypic Culture of Fetal Lung Type II Alveolar Epithelial Cells: Applications to Pulmonary Toxicology

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Techniques for isolation and culture of fetal Type II alveolar epithelial cells, as well as the morphologic and biochemical characteristics of these histotypic cultures, are described.

Type II alveolar epithelial cells can be isolated from fetal rat lungs and grown in an organotypic culture system as described in this review. The fetal Type II cells resemble differentiated rat Type II cells in morphology, biochemistry, and karyotype as they grow in culture for up to 5 weeks. The cells of the mature organotypic cultures form alveolarlike structures while growing on a gelatin sponge matrix. The Type II cells also synthesize and secrete pulmonary surfactant similar in biochemical composition to that produced *in vivo*. This system has been used to study the effects of hormones on surfactant production and composition. The organotypic model has many potential applications to the study of pulmonary toxicology.

Introduction

The alveolar region of the lung is lined by a continuous epithelium consisting of two morphologically and functionally distinct cell types. These are the Type I cell, a squamous cell which acts as a protective barrier while allowing gas exchange, and the Type II cell, a cuboidal cell with characteristic inclusion bodies or lamellar bodies in the cytoplasm. Pulmonary surfactant is synthesized by the Type II cell and stored in these inclusion bodies (*1*). Surfactant is a lipoprotein complex lining the alveolar epithelium (*2–4*) and reducing surface tension (*5*); the low surface tension prevents alveolar collapse (*6,7*). Surfactant may also aid in preventing pulmonary edema (*8,9*) and may enhance the bactericidal action of macrophages and aid in the clearance of particulates (*10,11*). In addition to producing surfactant, the Type II cell also serves as the stem cell for the Type I cell (*12,13*), forming the basis for the renewal of the alveolar epithelium.

Pulmonary disorders frequently originate in the alveolar region of the lung. In human infants, a deficiency in surfactant is thought to be a cause of the respiratory distress syndrome (*5*). Both Type I and Type II cells are the targets of many toxic and carcinogenic agents in experimental animals as well as in humans (*14*). These toxic agents include gases such as NO_2 (*15*), ozone (*15,16*) and oxygen (*17*); metals such as cadmium (*18*) and metal salts (*19*); and drugs and chemicals such as BHT (*20*), bleomycin (*21*), lysolecithin (*22*), the hypocholesteremic drug AY9944 (*23*), Freund's adjuvant (*24*), and trypsin (*25*).

The lung contains numerous cell types (*26*). Therefore, in order to study the effects of toxic substances on the Type II cell and on surfactant production, it is advantageous to work with a population enriched in Type II cells maintained in tissue culture. Subtle effects on metabolism of the Type II cell will be more apparent in such a system. In addition, it is possible to control the environment of the Type II cell more closely when a tissue culture system is utilized. An organotypic model of fetal alveolar lung cells has been developed (*27–29*) in which the morphology and biochemical functions of the Type II pneumonocyte can be maintained *in vitro* for a period of several weeks. This model has been used to study the biochemistry of surfactant production (*30–32*) and could well be applied to the study of pulmonary toxicology. Although there are several cell culture systems for the study of Type II pneumonocytes (*33–43*), the organotypic model has certain advantages. It consists of an enriched population of Type II cells which grow in an alveolar rather than a monolayer pattern permitting cell to cell contacts and interactions

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similar to the *in vivo* situation. In addition, the fetal Type II cells present initially in culture continue to differentiate and mature into a population of metabolically active Type II alveolar pneumonocytes that synthesize and secrete pulmonary surfactant.

The dissociation of fetal tissue into single cells and its subsequent reaggregation into histotypic structures was first described by Moscona (*44,45*). Histotypic reaggregation has been described for fetal heart (*46*), brain (*47*), kidney (*48*) and liver (*49*). When dissociated, fetal rat lung cells reaggregate to form histotypic structures in a gelatin sponge matrix (Gelfoam) (*28,29*). Initially, these histotypic structures resemble the epithelial tubules of the fetal lung, containing pyramidally shaped columnar cells with glycogen accumulations in the cytoplasm (*50*). As they mature in culture, the cells in the histotypic structures begin to resemble differentiated Type II pneumonocytes which form alveolarlike structures (ALS) (*28,29*). Surfactant is synthesized, packaged into lamellar bodies and released into the lumina of the ALS by the Type II pneumonocytes present in the organotypic culture system (*32*). This surfactant can be collected by sucrose density centrifugation (*51*). Analysis of the phospholipids of the surfactant isolated from the organotypic cultures indicates that they are similar to the phospholipids present in whole lung (*32*).

In this paper we describe the techniques for isolation and culture of the fetal Type II alveolar epithelial cells as well as the morphologic and biochemical characteristics of these histotypic cultures. In addition, the response of this system to certain hormones is described.

Methods

It has been demonstrated that cellular reaggregation is dependent upon the presence of cell surface receptors (*52–55*). Reaggregation of fetal alveolar epithelial cells into histotypic structures is dependent upon methods used for isolation and culture of the cells. The isolation and culture methods have been reported in detail previously (*28,29,56*) and are reviewed here. Fetuses are aseptically obtained from pregnant rats at days 16 through 19 of gestation, and the lungs are removed, washed and minced. The lung tissue is then dissociated by a series of enzymatic digestions. The enzyme mixture consists of trypsin, collagenase, and chick serum in Moscona's saline. The freshly isolated cells are centrifuged and the cell pellet is incubated at 37°C for 1 hr. This 1-hr incubation is necessary for the resynthesis of cell–ligand components on the cell membrane (*28*). The cells are then resuspended in medium (RPMI-1640 supplemented with 10% selected fetal bovine serum) and aliquots of 10^7 viable cells are inoculated onto Gelfoam sponges (Gelfoam, The Upjohn Co., Kalamazoo, MI) which have been hydrated with medium. The sponges are incubated for 1 hr in Petri dishes at 37°C in a humidifed atmosphere of 5% CO_2 in air in order to allow the cells to attach to the Gelfoam matrix. Culture medium is then added to the dishes and they are incubated under the same conditions. After 2 days, the cultures are placed on a rocker platform set at 3 cycles/min. The culture medium is replaced every 2 days.

The ALS can be identified and assessed by staining with tetrazolium chloride (TTC). The cultures are incubated at 37°C with 0.05 to 1.0% of 2,3,5′-triphenyl tetrazolium chloride for 40 to 50 min. They become bright red in color as oxidative enzyme systems reduce the colorless tetrazole to the insoluble red-colored formazan. This staining technique has two specific applications to the organotypic model. It allows localization of the cells in the Gelfoam matrix such that they can be easily processed for morphological studies. In addition, TTC staining indicates the viability of the cells (*57*). Quantitative viability assays may be done using acetone-extracted formazan (*58*). Cells killed by glutaraldehyde fixation, freeze-thawing, or heating do not stain with TTC (*57*). TTC staining is routinely used to screen cultures before processing them for biochemical studies of surfactant (*57*). The technique might also be used to screen the effects of various toxic agents upon the Type II cells in the organotypic cultures.

Characteristics of the Culture System

The enzymatically dissociated fetal lung cells are initially in a monodisperse condition. They begin to reaggregate *in vitro* within 24 hr and within 2 days they form alveolarlike structures (ALS) within the Gelfoam matrix (*28*). These ALS can be maintained for 5 weeks in culture (*28*). Cells from younger fetuses (16 to 17 days of gestation) form ALS earlier than cells from 19– to 20–day fetuses (*31*). Initially, ALS are lined by a single layer of undifferentiated epithelial cells and are 12 to 50 μm in diameter (*28*). With time, these cells comprising the ALS differentiate into mature Type II alveolar epithelial cells while the ALS increase in diameter (*31*). The increase in size is most likely due to the addition of cells which were previously free, or coalescing of separate ALS, or a combination of the two since mitotic activity in these lung cells is minimal (*31*). Figures 1 and 2 demonstrate the appearance of ALS within the Gelfoam matrix after 7 and 22 days in culture.

Initially, the alveolarlike structures are surrounded by a loose meshwork of fibroblastlike cells. With time in culture these fibroblastlike cells condense into a dense mesenchymal matrix which may be one to several layers of cells in thickness (*31*). This mesenchymal matrix may be quite important to the functional and morphological development of the Type II cells of the ALS. Smith (*59*) has described a fibroblast–pneumonocyte factor which fetal lung fibroblasts produce upon glucocorticoid stimulation. This factor induces the fetal lung to mature *in vitro* without a corresponding decrease in growth rate. In addition, Master (*60*) has demonstrated that, in the mouse lung in organ culture, the amount of mesenchyme in the system regulates growth and differentiation of the epithelial cells.

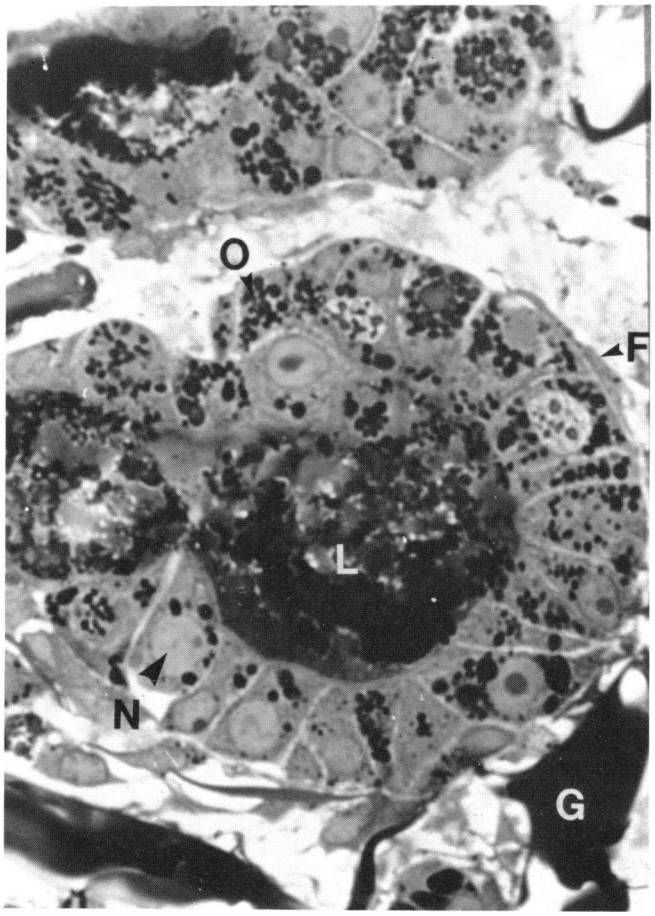

FIGURE 1. Photomicrograph of an alveolarlike structure after 22 days *in vitro*. Type II cells in the ALS have a euchromatic nucleus (n) with osmiophilic inclusion bodies in their cytoplasm (O). The Type II cells surround the lumen (L) of the ALS. Fibroblasts (F) are present at the periphery of the ALS between the ALS and the Gelfoam matrix (G). Magnification: ×1,000.

The undifferentiated cells that originally comprise the ALS contain abundant glycogen. This glycogen is lost as these cells differentiate into Type II alveolar epithelial cells. Like Type II cells *in vivo*, the Type II cells in organotypic culture are cuboidal with apical microvilli and a euchromatic nucleus (*1*). They contain osmiophilic lamellar bodies resembling those formed *in vivo* (*35,61*). The lamellar bodies are released into the lumina of the ALS where they form tubular myelin figures (*31*). Desmosomes and tight junctions join the apical surfaces of cells in the ALS; other regions of contact between the cells are interdigitating folds (*28*). This morphology of the Type II cell is maintained for 5 weeks in culture (*31*). Most of the cells in the culture exhibit a diploid rat karyotype of 42 chromosomes (*31*).

In addition to Type II cells, a small number of other pulmonary cell types may be found in these cultures. Occasional ciliated cells may line the ALS after 1 week in culture. Nonciliated cells and cells resembling endocrine cells may also be observed infrequently (*31*). After 15 days in culture, squamous epithelial cells that resemble the Type I cell are found in the ALS (*28*). It is logical to assume that these squamous cells are, in fact, Type I pneumonocytes because the Type II cell is the stem cell for the Type I cell *in vivo* (*13*).

The organotypic cultures, after 1 week in culture, are used routinely to study the synthesis of surfactant (*32*). Surfactant is isolated by the method of Frosolono (*51*) and has been found to be similar in composition to that isolated from adult rat lung (*32*). Table 1 gives proportions of the major surfactant components in whole lung and in organotypic cultures. It should be noted that there may be some differences in phospholipid composition of fetal organotypic Type II cells and adult whole lung Type II cells because phospholipid composition of surfactant does appear to change during lung development (*62*) as does esterase activity (*63*). Any cellular material which is not isolated with the surfactant fraction is essentially equivalent to the lamellar body fraction while the residual fraction approximates the microsomal fraction (*32*).

The most abundant phospholipid found in the surfactant fraction of the organotypic culture is phosphatidylcholine (PC) (72%); this percentage is similar to that found in adult rat lung (69%). The residual fractions of lung contain less PC than the surfactant fractions (51% in the organotypic culture and 46% *in vivo*) (*32*). The PC of the surfactant fraction of the organotypic culture is highly saturated, containing palmitate (79%), myristate (9%), and stearate (8%) as the primary fatty acids (*57*). These fatty acids are found in similar concentrations in PC from adult whole rate lung (*64*). The surfactant fraction contains relatively little phosphatidylethanolamine (PE) and spingomyelin *in vitro* (9% and 7%, respectively) as *in vivo* (10% and 5%, respectively). The residual fractions in both organotypic cultures and *in vivo* contain a greater proportion of PE and sphingomyelin than do the surfactant fractions (*31*).

Exogenous glucose has been measured as it is metabolized by the organotypic cultures. The glucose may be utilized by the Type II cell for the synthesis of both surfactant and residual PC. Residual PC incorporates three times more ^{14}C-glucose than does surfactant PC. The uptake of ^{14}C-glucose into PC is concentration-dependent (*32*) and may be stimulated by insulin exposure *in vitro* (*65*).

In order to demonstrate that these organotypic cultures actually synthesize surfactant, they have been incubated with radioactive precursors of surfactant such as ^{3}H-palmitate, ^{14}C-choline, or ^{3}H-leucine. Palmitate is incorporated in a linear manner into both surfactant and residual fractions for 3 hr after which five times more is incorporated into residual than surfactant fractions. Choline is incorporated into surfactant PC in a linear fashion and into residual PC in a biphasic manner. Incorporation of leucine into the protein of surfactant fraction is linear for 8 hr (*32*).

Certain nonspecific esterases are characteristic of mouse and rat lung homogenates (*66*) as well as mouse pulmonary lavage fluid (*66*), lamellar body fractions

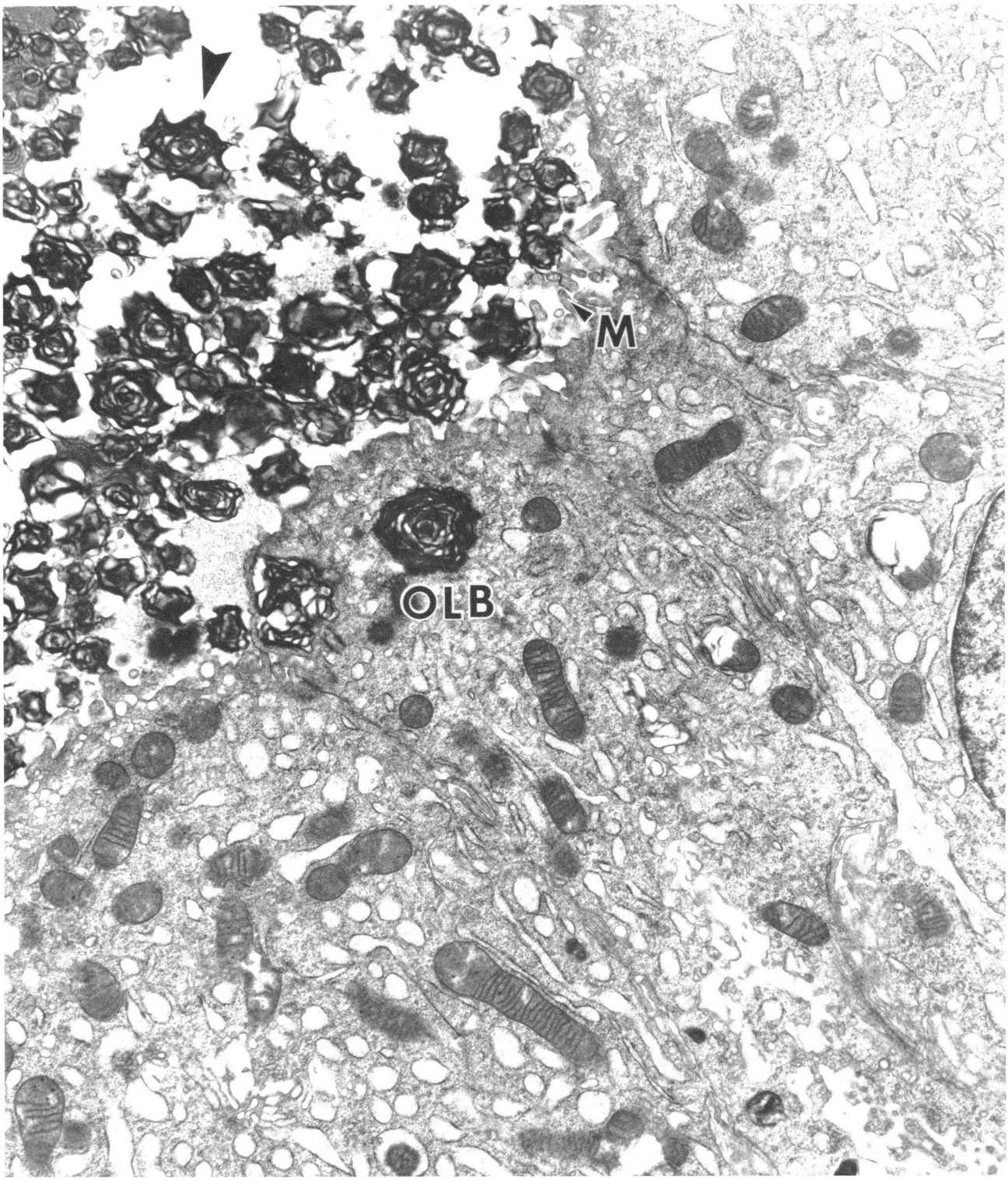

FIGURE 2. Electron micrograph of a portion of an alveolarlike structure after 7 days *in vitro*. Portions of four Type II cells are present. Osmiophilic lamellar bodies (OLB) are present in the cytoplasm of the cells and also in the lumen (arrowhead) of the ALS. Microvilli (M) are present on the apical surface of the Type II cells. Magnification: ×23,200.

Table 1. Phospholipid composition of adult rat lung and organotypic cultures from fetal rat lung.

	Rat lung[a]		Organotypic cultures[b]	
	Surfactant, %	Residual, %	Surfactant, %	Residual, %
Phosphatidylglycerol	6.7 ± 0.3	2.8 ± 0.2	4.0 ± 0.5	2.2 ± 0.2
Phosphatidylethanolamine	9.8 ± 0.4	23.6 ± 0.7	8.8 ± 1.8	14.2 ± 0.8
Phosphatidylserine and phosphatidylinositol	6.1 ± 0.5	10.5 ± 0.7	5.6 ± 0.5	7.5 ± 0.6
Phosphatidylcholine	68.8 ± 1.3	46.2 ± 1.4	71.5 ± 3.0	50.6 ± 1.7
Sphingomyelin	5.0 ± 0.2	12.5 ± 0.5	6.6 ± 0.5	19.9 ± 1.7
Others	3.6 ± 0.2	4.3 ± 0.5	3.7 ± 0.4	7.6 ± 0.7

[a] Mean ± SEM; $n = 30$.
[b] Mean ± SEM; $n = 44$.

(*66*), and isolated Type II cells (*27,66*). The lamellar body of the Type II cell is the major site of surfactant-associated esterase activity within the lung (*66*). In the Type II cells of the organotypic cultures, the lamellar body is also the major site of esterase activity (*30*). The nonspecific esterases of the cultured Type II cells resemble those of whole rat and mouse lung homogenates and pulmonary lavage fluid in biochemical, cytochemical, and electrophoretic characteristics (*30*). This finding yields additional evidence that organotypic cultures of Type II cells retain surfactant-associated functions of Type II cells *in vivo*.

The organotypic model has already been utilized to study the effects of certain hormones upon production of surfactant. *In vivo*, glucorticoids are known to accelerate fetal lung maturation and surfactant production although the mechanism of action for this effect is not known (*5*). In the organotypic model, dexamethasone treatment produces a dose-dependent increase in the synthesis and cellular content of surfactant PC and surfactant PG but has no effect on the content of synthesis of residual PC and residual PG (*67*). Thyroid hormones have also been suggested to accelerate the development of the fetal lung (*68,69*). Triiodothyronine (T3) has been found to increase the PC content of both surfactant and residual fractions of organotypic cultures; residual PC increases immediately after exposure to T3, while surfactant PC is maximal after 24 hr of exposure to the hormone (*70*). Other hormones which have been implicated as accelerators of fetal lung maturation *in vivo* include estrogens (*71*) and prolactin (*72*).

Application to Pulmonary Toxicology

There are several areas of pulmonary toxicology which might be explored more thoroughly with the utilization of the organotypic model. One of the advantages of this system is that it will allow a more accurate knowledge of the dose of an agent delivered to the Type II cell than can be ascertained *in vivo*. In the animal, factors such as temperature changes, hypoxia, pH changes, damage to the vasculature, and clearance by blood, alveolar fluid, and lymph will all have a potential effect on the dose received by the Type II cells (*73*). In addition, certain agents, such as SO_2 and water-soluble aerosols which are difficult to deliver to the alveolar region in the whole animal because they impact in the upper airways, could be employed relatively easily with this model.

From studies utilizing lung homogenates, it is known that changes in pulmonary enzymes occur after injury (*74*). However, the mechanisms responsible for these changes are not known. A frequently studied example of this is the phenomenon of tolerance to oxygen or ozone (*74,75*) which is thought to involve changes in the enzyme activity of Type II cells (*39*). A fetal organ culture system similar to the organotypic model has been used to study oxygen toxicity (*76*). The organotypic model might be applied to elucidate the mechanism of this phenomenon more fully.

In vivo studies indicate that many pulmonary toxicants decrease the synthesis of surfactant. This decrease in surfactant has been proposed as a sensitive screen for pulmonary irritants because the decrease often occurs before morphological changes are observed (*77*). Gasoline vapors decrease surfactant in rat lungs (*77–79*), as do trichloroethylene, carbon tetrachloride and cigarette smoke (*77*). These changes can be detected as early as 5 days after exposure (*77*). Fluorocarbons such as anesthetic agents (*80*), increased concentrations of oxygen (*81*), and the ingestion of paraquat (*82*) also decrease surfactant production. The effect of irritants on surfactant production could be studied further with the use of the organotypic model system.

Viral respiratory infections may act additively with chemical compounds in causing lung damage or carcinogenesis (*83,84*). There is also evidence that pulmonary infections decrease the number of lung tumors experimentally induced (*85,86*). The organotypic system has been used to study the response of Type II cells to infection with certain respiratory viruses (*87*). It might also be utilized to study the effect of interactions of viruses with chemicals on the Type II cell.

Finally, it should be noted that lung tumors do arise from Type II cells in humans (*88–90*), forming alveolar cell cancers, bronchiolar–alveolar cancers, and pulmonary adenomatosis. In humans, 1.5 to 5.0% of pulmonary neoplasms are derived from Type II cells (*38*).

Type II cells are also the origin of lung cancers in animals such as sheep (*91,92*) and mice (*93,94*). The mouse adenoma model has been proposed as another screening method for lung carcinogens (*94*). The organotypic model should be considered as an additional means for assessing the process of malignant transformation of the alveolar Type II cell. This model might prove especially helpful in estimating doses of carcinogens necessary for various preneoplastic and neoplastic changes in the cells.

Summary and Conclusions

The Type II cell plays a part in the response of the lung to many toxic and carcinogenic agents as well as being involved in such diseases as the respiratory distress syndrom of the newborn. In addition, the Type II cell is the stem cell of the alveolar epithelium and serves as a reservoir for any damaged Type I cells. Thus, any new model for the study of the Type II cell and its functions can be very useful in understanding mechanisms of injury to the peripheral lung. The fetal lung organotypic culture system is such a model for it possesses many advantages inherent in a tissue culture system, and the Type II cells also retain many characteristics of Type II cells *in vivo*. The method for establishing a fetal organotypic Type II culture depends upon the histotypic reaggregation of fetal alveolar pneumonocytes after the fetal lung has been enzymatically digested. The pneumonocytes grow in alveolarlike structures upon a layer of fibroblasts. After a few days in culture, the organotypic Type II cells are similar in morphology to Type II cells *in vivo*. Surfactant produced by the organotypic cells has been characterized biochemically and reflects the composition of surfactant produced by the whole rat lung.

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